CHAPTER-I

1. INTRODUCTION

Helicobacter pylori is a gram negative, spiral-shaped microaerophilic organism that colonizes gastric mucosa. It is an etiologic agent of acute or chronic gastritis and a predisposing factor in peptic ulcer, gastric cancer (Parsonnet *et al.*, 1991), B-cell mucosa-associated lymphoid tissue (MALT) lymphoma (Wotherspoon *et al.*, 1991), non-Hodgkin's lymphoma (Parsonnet *et al.*, 1994) and Menetrier's disease (Bayerdorffer *et al.*, 1994). International Agency for Research on Cancer has recognized it as a group 1 carcinogen (IARC, 1994).

H. pylori seems to be acquired during childhood. Low socio-economic conditions, over-crowding, poor sanitation and close contact with infected individuals appear to be the prominent risk factors. The location of *H. pylori* infection and the basic need of this bacterium for gastric-type mucosa for in vivo proliferation suggest ingestion as the most likely means of acquiring *H. pylori*. However, whether *H. pylori* reaches the oral cavity via the gastro-oral, oral-oral, or fecal-oral route remains uncertain (Mitchell, 2001). Although infected individuals often have histological evidence of gastritis, the vast majority of infections are asymptomatic.

H. pylori has an estimated prevalence of about half the world's population, possibly reaching up to 70% in developing countries and 20–30% in industrialized countries. Infections seem to be more common with age (IVR, 2008). In the developing world the infection is common and in some areas virtually everyone is infected. Acquisition of *H. pylori* infection has decreased radically in the developed world; therefore, the prevalence is higher in the elderly than in the young (Malaty, 2007). During childhood transient infection may be common that may clear later. It may be due to consumption of antibiotics for other reasons. There are significant differences in the prevalence of *H. pylori* infection both within and between countries. The infection is worldwide and infects both males and females (Mitchell, 2001).

H. pylori infection can be diagnosed by invasive methods (that require gastric biopsies) as well as non-invasive methods. The selection of appropriate test depends upon clinical setting (Howden and Hunt, 1998). Patients with alarming symptoms, such as anemia, GI bleeding, weight loss and elderly patients (more than 50 years of age) undergo endoscopy for the diagnosis of *H. pylori* infection. The test of first choice is a urease test on an antral biopsy specimen (Howden and Hunt, 1998). It allows cheap and rapid detection of *H. pylori* urease activity with a sensitivity of 79 to 100 percent and a specificity of 92 to 100 percent (Graham and Qureshi, 2001). If the urease test is negative, additional biopsy specimens can be sent for histopathology. Culture of *H. pylori* with antibiotic sensitivity testing is not routinely performed but is recommended after the failure of second-line therapy (Suerbaum and Michetti, 2002).

Noninvasive methods include the urea breath test (UBT), serologic tests, stool antigen assays, urine antibody assays and salivary antibody assays. Both ^{13}C and ^{14}C urea breath tests have been widely used in patients both before and after *H. pylori* eradication therapy. It is based on the urease activity of *H. pylori* and detects active infection with high sensitivity and specificity. Serologic testing of *H. pylori* is cheap and widely used for the diagnosis of *H. pylori* infection in patients before treatment. Although approved laboratory-based tests have sensitivity and specificity similar to those of the urea breath tests, inconsistent results have been reported with some office-based tests (Suerbaum and Michetti, 2002). Stool antigen tests for *H. pylori* provide an alternative to the urea breath test, with a sensitivity of 89 to 98 percent and a specificity of over 90 percent (Graham and Qureshi, 2001).

Gastritis occupies the fourth position (2.17%) among the top ten OPD diseases in Nepal. Further its occurrence is reported more in mountainous region than in Hilly and Terai region. Cases of peptic ulcer and gastric cancer have also been reported (DoHS, 2006/2007). There is limited information about the prevalence of *H. pylori* infection in Nepal and the true incidence of the peptic ulcer and gastric cancer in Nepal is not reported yet. However, studies have been carried out in different subpopulations. In

Nepal, the diagnosis of *H. pylori* infection is chiefly based on histopathology but results of rapid urease tests have also been reported (Rai *et al.*, 2006; Makaju *et al.*, 2006).

Current treatment regimens for *H. pylori* include varied combinations of antibiotics, proton pump inhibitors and histamine 2 receptor antagonists. The major antibiotics used for this purpose are metronidazole, amoxycillin, clarithromycin and tetracycline. Eradication rates range from 61% to 94% depending on the regime used. Antibiotic resistance and patient non-compliance are the two major reasons for treatment failure (CDC, 2006).

Since *H. pylori* infection leads to various complications like peptic ulcer and gastric cancer, timely diagnosis and treatment is necessary. This study was performed with the objective to determine the prevalence of *H. pylori* infection among the symptomatic cases in Bir Hospital, a referral hospital and to provide data regarding antibiotic resistance pattern of *H. pylori* against commonly used antibiotics. These findings might be helpful to the clinicians in treatment of *H. pylori* infection.

CHAPTER-II

2. OBJECTIVES

2.1 General Objective

To determine the prevalence of *H. pylori* among dyspeptic patients attending Gastroenterology Department, Bir Hospital.

2.2 Specific Objectives

1. To perform RUT for the rapid diagnosis of *H. pylori* from antral biopsy samples.
2. To isolate *H. pylori* from biopsy sample and to perform biochemical tests.
3. To evaluate the association of *H. pylori* infection with different endoscopic findings.
4. To determine the sensitivity, specificity, PVP and PVN of RUT using culture as gold standard in the diagnosis of *H. pylori* infection.
5. To evaluate the antibiotic susceptibility pattern of *H. pylori* isolates.

CHAPTER-III

3. LITERATURE REVIEW

3.1 Anatomy of stomach and duodenum

Stomach, also called the gaster is a muscular bag and most distensible part of the digestive tube. It is connected above to the oesophagus and below to the duodenum. It lies obliquely in the upper and left part of the abdomen, occupying the epigastric, umbilical and left hypochondriac regions. It is a J-shaped organ and is about 10 inches long. It has two orifices, two curvatures and two surfaces. The cardiac orifice is joined by the lower end of the oesophagus. The pyloric orifice opens into the duodenum. The lesser curvature is concave and forms the right border of the stomach. At the lower end of the lesser curvature is the angular notch. The greater curvature is convex and forms the left border of the stomach. At the upper end of the greater curvature is the cardiac notch. The anterior surface faces forwards and upwards. The posterior surface faces backwards and downwards (Chaurasiya, 1999).

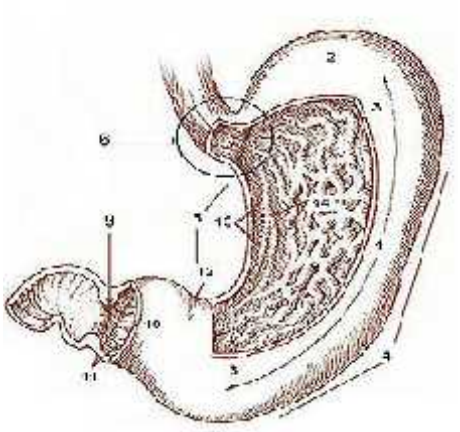


Figure 1: Anatomy of the Stomach (Wikipedia)

1. Body	9. Pyloric sphincter
2. Fundus	10. Pyloric antrum
3. Anterior wall	11. Pyloric canal
4. Greater curvature	12. Angular notch
5. Lesser curvature	13. Gastric canal
6. Cardia	14. Rugal folds

The stomach is divided into four major regions.

i. The cardia, near the cardiac orifice

ii. The fundus, dome-shaped part above the level of the cardiac orifice

iii. The body or corpus, the large central portion

iv. The pyloric part, comprised of pyloric antrum and pyloric canal

The stomach wall consists of four layers, from inside to outside.

1. Mucosa consists of columnar epithelium, lamina propria composed of loose connective tissue having gastric glands below and the muscularis mucosae, a thin layer of smooth muscle.
2. Submucosa lies under the mucosa and consists of fibrous connective tissue and Meissner's plexus.
3. Muscularis externa lies under the submucosa and consists of three layers of smooth muscles viz; inner oblique layer, middle circular layer and outer longitudinal layer.
4. Serosa is under the muscularis externa and consists of layers of connective tissue continuous with the peritoneum.

When the stomach is empty the mucous membrane forms longitudinal folds or rugae, and when full the rugae disappear and the surface appears smooth and velvety. There are numerous small depressions on the mucosal surface called gastric pits where the gastric glands open (Chaurasiya, 1999). Four major types of secretory epithelial cells are present at different layers of these glands, viz.
1. Mucous cells that secrete alkaline mucus
2. Parietal or oxyntic cells that secrete hydrochloric acid and intrinsic factor
3. Chief or zymogenic cells that secrete pepsinogen
4. Enteroendocrine cells that secrete the hormone gastrin.

In *H. pylori* infected patients there is an increase in fasting and in meal-stimulated serum gastrin levels (Graham and Qureshi, 2001).

The duodenum is about 25 cm long, C-shaped and begins at the pyloric sphincter. It consists of four parts (superior, descending, horizontal and ascending parts). The superior (first) part is one of the commonest sites for peptic ulcer, possibly because of direct exposure of this part to the acidic contents reaching it from the stomach (Chaurasiya, 1999). The gastro-duodenal mucosa is able to resist auto-digestion by high concentrations of hydrochloric acid and pepsin with the help of protective factors such as mucus, bicarbonate, epithelial cell barrier, blood flow and prostaglandin protection.

3.2 Infections of stomach and duodenum

3.2.1 Gastritis

Injury to the gastric mucosa is associated with epithelial cell damage and regeneration. The term gastritis denotes inflammation associated with mucosal injury. Most classification systems distinguish acute and chronic disease. Acute inflammation is usually associated with neutrophilic infiltration while chronic inflammation is usually characterized by mononuclear cells, chiefly lymphocytes, plasma cells and macrophages. The mechanisms of mucosal injury in gastritis are thought to be an imbalance of aggressive factors like acid production or pepsin and defensive factors like mucus production, bicarbonate and blood flow (El Salvador Atlas of Gastrointestinal Video Endoscopy). Sydney classification of gastritis has classified gastritis as acute, chronic and special forms on the basis of histomorphologic criteria (Misiewicz, 1991).

a. Acute gastritis

Acute gastritis is a group of disorders that provoke inflammatory changes in the gastric mucosa. It may involve a region (eg, antral gastritis) or the entire stomach (pangastritis).

Acute gastritis may produce no symptoms or may show dyspepsia, lack of appetite, nausea or vomiting and sometimes be severe causing gastrointestinal (GI) bleeding with melena or hematemesis. It may be caused by prolonged use of certain drugs (NSAIDs such as aspirin and ibuprofen); alcohol; bacterial (*H. pylori*), viral, and fungal infections; acute stress (shock); radiation and direct trauma (El Salvador Atlas of Gastrointestinal Video Endoscopy).

The gastric mucosa protects itself from gastric acid with a layer of mucus, the secretion of which is stimulated by certain prostaglandins. NSAIDs block the function of cyclo-oxygenase 1, which is essential for the production of these prostaglandins. Aspirin directly irritates the gastric mucosa and reduces prostaglandin. Although short term use of these drugs is not harmful, regular use can lead to gastritis. Alcohols are direct irritants of the stomach.

H pylori gastritis typically starts in the antrum, causing intense inflammation, and may extend to involve the entire gastric mucosa. At endoscopy the inner lining of the stomach (mucosa) may appear swollen, reddened and inflamed. There may be small, shallow erosions (breaks in the surface lining) or even tiny areas of bleeding from the mucosa.

b. Chronic gastritis

Chronic gastritis is characterized by chronic inflammation of the gastric mucosa. It is usually associated with *H. pylori,* bile reflux, non-steroidal anti-inflammatory drugs (NSAIDs), autoimmunity and allergic response.

H pylori associated chronic gastritis progresses with two distinct topological patterns that have different clinical consequences. Antral predominant gastritis is mostly limited to the antrum that may later develop into peptic ulcer. Multifocal atrophic gastritis involves the corpus and antrum with progressive development of gastric atrophy and intestinal metaplasia. Individuals who develop gastric carcinoma and gastric ulcers usually demonstrate this pattern of gastritis (El Salvador Atlas of Gastrointestinal Video Endoscopy).

Most of the cases are asymptomatic and do not develop significant clinical complications. Only, some individuals who carry additional risk factors may develop peptic ulcer, gastric mucosa–associated lymphoid tissue (MALT) lymphoma, or gastric adenocarcinoma.

3.2.2 Peptic ulcer

Peptic ulcer is also known as *ulcus pepticum*, PUD or peptic ulcer disease. Ulcers in the stomach and duodenum are called peptic ulcers. Most peptic ulcers arise in the duodenum rather than in the stomach.

Types of peptic ulcers:

Type I: Ulcer along the lesser curve of stomach
Type II: Two ulcers - one gastric, other duodenal
Type III: Pre-pyloric ulcer
Type IV: Proximal gastro-esophageal ulcer
Type V: Anywhere along gastric body, NSAIDS induced

The major symptoms of peptic ulcer include bloating and abdominal pain, waterbrash, nausea and vomiting, loss of appetite and loss of weight, hematemesis and melena. Gastrointestinal perforation, bleeding and gastric outlet obstruction are the complications.

H. pylori is the causative agent of most peptic ulcers. After colonizing antrum, it interferes with gastrin regulation. Gastrin secretion may be decreased (most cases) leading to hypochlorhydria or achlorhydria. Sometimes, it may be increased that stimulates acid secretion which in turn erodes the mucosa and ultimately leads to ulcer formation.

Ulcers may also be caused or exacerbated by drugs, smoking, stress etc. Glucocorticoids lead to atrophy of all epithelial tissues. Smoking leads to atherosclerosis and vascular spasms, causing vascular insufficiency and promoting the development of ulcers through ischemia. Chronic stress has been strongly associated with peptic ulcer, and a combination of chronic stress and irregular meal is a significant risk factor. Blood group O is associated with duodenal ulcer. Gastrinomas (rare gastrin-secreting tumors) cause multiple and difficult to heal ulcers.

3.2.3 Gastric cancer

Gastric cancer is the second most frequent cause of cancer-related death. It is one of the most common malignancies in the world, although the incidence and mortality rate have been decreasing in recent decades. The association of *H. pylori* with gastric cancer has attracted many researchers as the International Agency for Research on Cancer (IARC),

a subordinate organization of the World Health Organization (WHO), identified *H. pylori* as a group 1 carcinogen in 1994 (IARC , 1994).

Most cases of gastric cancer develop on gastric mucosa with multifocal atrophic gastritis, usually with extensive intestinal metaplasia, suggesting that intestinal metaplasia and gastric atrophy are premalignant lesions of the stomach. As these lesions are intimately associated with chronic *H. pylori* infection, they provide a strong link of *H. pylori* infection with gastric carcinogenesis. There is high prevalence of *H. pylori* antibody in countries which have high morbidity of gastric cancer (Asaka *et al.*, 2001).

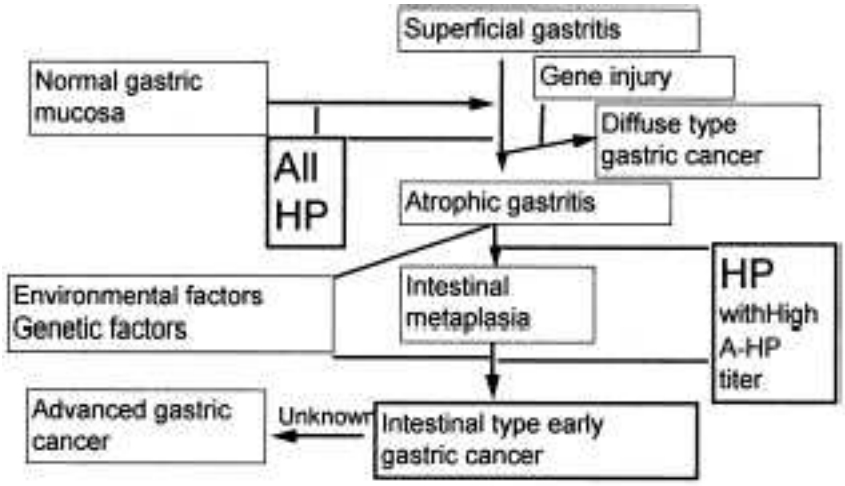


Figure 2: Association of *H. pylori* infection with development of gastric cancer (Asaka *et al.*, 2001)
HP: *H. pylori*

H. pylori might cause gastric carcinomas by two main events: (i) collateral damage from inflammatory by-products causing mutational events in gastric epithelial cells and (ii) direct effects on gastric epithelial cells by *H. pylori* or released bacterial products at different levels.

Environmental factors (e.g., diet) and genetic factors may also participate in this progression to intestinal metaplasia. There is a sharp increase in incidence of gastric cancer after the age of 50. Diets containing large amounts of smoked food, salted fish,

meat and pickled vegetables increase risk of gastric cancer. Gastric cancers are more likely to develop in people who had undergone gastrectomy to treat non-cancerous diseases such as ulcers. People with type 'A' blood group and several first degree relatives suffering from gastric cancer are more likely to develop this disease (American Cancer Society).

3.3 *Helicobacter pylori*

3.3.1 History

Spiral-shaped bacteria were observed in the gastric mucosa by German scientists in 1875 but could not be cultured and were ultimately forgotten. Again in 1899, Professor Walery Jaworsky found spiral bacteria in sediments of human gastric washings which he called *Vibrio rugula* and became the first person to suggest their possible role in the pathogenesis of gastric diseases. Later, several studies demonstrated the presence of curved rods in the stomach of many patients with peptic ulcers and stomach cancer (Wikipedia, 2009). However, the interest faded away when an American study could not observe the bacteria in 1180 gastric biopsies (Palmer, 1954).

The interest re-emerged with the visualization of the bacteria in the stomach of gastric ulcer patients (Steer, 1975). Later in 1979, Australian pathologist Robin Warren, observed similar bacteria in the gastric mucosa who did further research with Australian physician Barry Marshall. They cultured it from gastric mucosa in 1982 and confirmed its association with most stomach ulcers and gastritis (Marshall and Warren, 1984). After isolation and identification in 1983, it was first named *Campylobacter pyloridis* due to its location and the resemblance to *Campylobacter* which was later changed to *Campylobacter pylori*. Again it was found that the bacterium differed from *Campylobacter* in important features such as flagellum morphology, fatty acid content and 16S rRNA sequence (Andersen and Wadstrom, 2001) and the name was changed to *Helicobacter pylori*, as it represented its own genus (Goodwin *et al*, 1989). *Helicobacter* reflects the two morphological forms of the organism, helical *in vivo* but often rod-like *in vitro.*

After its discovery, numerous research groups verified the association of *H. pylori* with gastritis and ulcers. In 1994, the National Institutes of Health (USA) published a view stating that most recurrent duodenal and gastric ulcers were caused by *H. pylori* and recommended that antibiotics be included in the treatment regimen (NIH Consensus Statement). Warren and Marshall were awarded the Nobel Prize in Medicine in 2005 for their work on *H. pylori* (The Nobel Prize in Physiology or Medicine 2005).

3.3.2 Habitat

The surface of human gastric mucosa is the major habitat of *H. pylori*. However, it has also been found in saliva, dental plaque, bile and faeces.

3.3.3 Morphology

H. pylori is a gram negative, spiral shaped bacterium with up to 4-8 unipolar sheathed flagella. It is about 3 μm long and 0.5-0.9 μm wide with a wavelength of about 2.6 μm. In agar cultures, the bacteria appear as single curved rods while spiral forms are less clear. It changes to coccal form on exposure to air within 1-2 hour at room temperature which fails to grow on subculture. These coccoid forms appear to be avirulent (Nachamkin and Skirrow, 1998).

3.3.4 Cultural characteristics, metabolism and growth requirements

H. pylori is a strict microaerophilic, capnophilic, fastidious organism that requires high humidity for growth. Supplements like blood, haemin, serum, starch or charcoal must be incorporated into the agar medium. Growth is best on media such as freshly prepared chocolate agar, or brain-heart infusion agar with 5% horse blood and 1% IsoVitalex. Strains grow in various liquid media supplemented with fetal calf or horse serum. Some strains grow in serum-free media, notably bi-sulphite free brucella broth. All strains grow at 37°C, some grow poorly at 30 and 42°C but none grow at 25°C. Colonies from primary culture at 37 °C usually take 3-5 days to appear and are circular, smooth and translucent. They are weakly haemolytic. Motility is weak or absent when grown on

agar. It is a strong producer of urease, catalase and alkaline phosphatase. All strains produce DNAse, leucine aminopeptidase and γ−glutamylaminopeptidase (Nachamkin and Skirrow, 1998).

3.3.5 Genetics

H. pylori has a small genome of about 1700 kb. The G + C content of their DNA is 35.2 ± 1.0 mol %. *H. pylori* is so genetically diverse that any two independent isolates can be distinguished by a variety of genetic analyses including restriction endonuclease analysis, restriction fragment length polymorphism and ribotyping. Plasmid transfer has not been reported in *H. pylori*, but plasmids have been detected in 48-58% of wild strains (Nachamkin and Skirrow, 1998).

3.4 Epidemiology

3.4.1 Prevalence and incidence

The prevalence of *H. pylori* infection varies widely by geographic area, age, race and socio-economic status.

It varies from country to country with large difference between developed and developing countries. The acquisition rate of *H. pylori* appears to be more rapid in developing than developed countries (Malaty, 2007). Studies showed the incidence, 0.4% per year for adults, but in children in developing and developed countries, 36% and 2.7% per year respectively (Neale *et al.*, 1995). The prevalence of *H. pylori* infection increases with age and most infections are acquired in childhood. This increase in prevalence with age may be attributable either to new acquisition of infection among the population or the effect of different birth cohorts, each with a different rate of acquisition in childhood (Malaty, 2007).

Although some studies have reported an excess of *H. pylori* in one gender versus the

other; no noteworthy gender differences exist in *H. pylori* prevalence overall. In United States, Whites have a substantially lower seroprevalence of *H. pylori* than either Blacks or Hispanics (Brown, 2000).

Different studies have suggested that *H. pylori* infection to be persistent, however, a spontaneous clearance has been reported in children (Mitchell, 2001). The possible explanation for transient infection may be the lower expression of *H. pylori* binding receptors in the gastric mucosa of children (Celik *et al.,* 1998) and treatment with antibiotics (Rothenbacher *et al.,* 1998).

3.4.2 Reservoirs

The well-established reservoir of *H. pylori* infection is human stomach. Other sources have also been hypothesized. However none have been definitely proven. Animals do not seem to play a significant role (Megraud, 2000).

3.4.3 Risk factors

Low socio-economic status has been found to be the major risk factor in *H. pylori* infection (Hopkins *et al*, 1993; Bani-Hani *et al.,* 2006; Malaty, 2007). Poor hygienic conditions and increased living density, either in families or institutions, especially during childhood are positively associated with *H. pylori* infection (Goodman *et al.,* 1996; Breuer *et al.,* 1996; Bani-Hani *et al.,* 2006). *H. pylori* positive family member has been demonstrated as a risk factor (Rothenbacher *et al.,* 1999; Goodman *et al.,* 2000). Close mouth-to-mouth contact has been identified as a risk factor based on studies reporting higher *H. pylori* prevalence among mothers chewing food for their babies and among Chinese immigrants using chop sticks and shared dishes (Mitchell, 2001). There is increased risk of *H. pylori* infection among endoscopists compared to the general population or to other medical staffs in the hospital (Lin *et al.,* 1994; Liu *et al.,* 1996). Similarly, higher risk of *H. pylori* infection has been found in dentists (Matsuda *et al.,* 2005). Further, a higher prevalence of *H. pylori* infection was demonstrated among

hospital workers with direct patient contact compared to those working in laboratories (Mastromarino *et al.,* 2005).

Host factors have also been identified as risk factors for infection. A study on twins had shown that genetic predisposition plays a role in the acquisition of *H. pylori* (Malaty *et al.,* 1994). The spread of infection may be promoted by low gastric acid secretion (Bjorkholm *et al.,* 2004). In addition, expression of blood group antigens that mediate bacterial adherence to the gastric mucosa has been suggested to be involved in the susceptibility to infection (Boren *et al.,* 1993).

3.4.4 Mode of transmission

The mode of transmission of *H. pylori* is poorly understood; no single pathway has been clearly identified. The most possible transmission pathway is person-to-person contact while transmission from animals and environment seems to occur less often (Megraud *et al*, 2000). Three possible routes of transmission from the stomach of one person to that of another have been described (Dunn *et al.,* 1997).

a. Iatrogenic route

It is the first and primary mode of transmission in which infection spreads through tubes or endoscopes that is inserted in the gastric mucosa of one patient to another (Akamatsu *et al.,* 1996). Occupationally acquired infections transmitted from a patient to staff member have also been reported, especially among endoscopists and gastroenterologists (Liu *et al.,* 1996; Lin *et al.,* 1994)

b. Faecal-oral route

The second possible route is faecal-oral. Isolation and identification of *H. pylori* from stool samples (Thomas *et al.,* 1992; Makristathis *et al.,* 1998; Oderda *et al.,* 2000) of infected patients suggest this route (Megraud *et al.,* 2000). Faeces-contaminated water may be a source of infection. Transmission via faeces-contaminated food has not been substantiated (Yvonne *et al.,* 2001).

c. Oral-oral route

The third possible route of transmission is oral-oral. Isolation and identification of *H. pylori* (Nguyen *et al.*, 1993; Namavar *et al.*, 1995; Ferguson *et al.*, 1993; Williams *et al.*, 1999) from saliva, dental plaque and refluxed gastric contents or vomits of infected patients suggest this route.

In developed countries the most likely route of transmission is oral-oral, probably via regurgigation and vomitus which occurs mainly in childhood. However, faecal-oral route is suggestive in developing countries due to poor hygiene and frequency of diarrhoeal diseases. Iatrogenic transmission by the endoscope or by gastric fluid may be found everywhere. Zoonotic transmission via cats, pigs, sheep and insects (houseflies) cannot be significantly involved in the transmission or may be important only in certain areas (Megraud *et al.*, 2000).

3.5 PATHOGENESIS

3.5.1 Virulence factors

Several virulence factors for gastric colonization, adhesion, tissue damage and survival have been identified in *H. pylori* (Table 1).

H. pylori is well-adapted to the gastric environment which otherwise, is invincible. Its unique features allow it to penetrate the mucus layer, attach the epithelia, evade the immune response, colonize and cause chronic inflammation.

After being ingested, the bacteria evade the bactericidal activity of the gastric contents and enter the mucus layer. Urease production and motility are essential for this purpose. Urease activity by ammonia production neutralizes the acidic environment and enhances the organism's acid tolerance in stomach. The enzyme activity is regulated by pH-gated urea channel UreI (Weeks *et al.*, 2000). The spiral shape and polar flagella aids in motility which in turn is essential for colonization.

Table 1: Virulence factors identified in *H. pylori*

Virulence factor	**Effect**
Colonizing factors	
Flagella	Active movements through mucin
Urease	Neutralization of acid
Adhesins	
BabA	the blood group antigen-binding adhesin
SabA	sialic acid binding adhesin
OipA	outer inflammatory protein
Tissue damaging factors	
Proteolytic enzymes	Glucosulfatase degrades mucin
Urease	Toxic effect on epithelial cells, disrupting cell tight junctions
Phospholipase A	Digest phospholipids in cell membranes
Alcohol dehydrogenase	Gastric mucosal injury
Disease Associated factors	
CagA	Related to ulcer and severe gastritis
Vacuolating cytotoxin (Vac A)	Damages the epithelium
dupA	duodenal ulcer promoting gene
iceA	Associated with peptic ulcer disease
Intracellular Survival	
Superoxide dismutase, Catalase	Prevent phagocytosis and killing
Coccoid forms	Dormant form
Other	
Lipopolysaccharide	Low biological activity
Blood group homology	Autoimmunity

Source: Andersen and Wadstrom, 2001

H. pylori binds to epithelial cells by multiple surface components. Several outer membrane proteins (OMP) like BabA, SabA and OipA mediate bacterial adherence to gastric epithelial cells (Figueiredo *et al.,* 2005).

BabA, a 78 kDa outer-membrane protein binds to the fucosylated Lewis B blood group antigen. An association between the presence of the OMP encoding genes and clinical outcome has been shown (Suerbaum and Michetti, 2002).

VacA (88 kDa toxin) is considered an important virulence factor in the pathogenesis of peptic ulcer and gastric cancer. This toxin can induce multiple cellular activities, including cell vacuolization, membrane channel formation, disruption of endosomal/lysosomal function, apoptosis and immunomodulation (Figueiredo *et al.,* 2005).

Most strains of *H. pylori* possess the *cag* pathogenicity island (*cag*-PAI), a 40 kb genome segment containing approximately 30 genes. *H. pylori* strains containing an intact *cag* pathogenicity island (*cag*-PAI) induce a strong inflammatory response than *cag*-negative strains (Figueiredo *et al.,* 2005). Several of these encode components of a type IV secretion apparatus that translocates the 120 kDa protein CagA into the host cell. After entering the epithelial cell, CagA is phosphorylated and binds to SHP-2 tyrosine phosphatase, leading to a growth factor-like cellular response and cytokine production by the host cell (Suerbaum and Michetti, 2002).

3.5.2 Host response to *H. pylori*

H. pylori causes chronic gastric inflammation in majority of infected persons. The inflammatory response initially consists of neutrophil infiltration, followed by T and B lymphocytes, plasma cells, and macrophages along with epithelial cell damage. As *H. pylori* rarely invades the gastric mucosa, the host response is triggered by the attachment of bacteria to epithelial cells. The pathogen binds to class II major histocompatibility complex molecules on the surface of gastric epithelial cells inducing their apoptosis. Further changes in epithelial cells depend on proteins encoded in the *cag*-PAI and on the translocation of CagA into gastric epithelial cells. *H. pylori* urease and porins may contribute to extravasation and chemotaxis of neutrophils (Suerbaum and Michetti, 2002).

The gastric epithelium of *H. pylori* infected persons has enhanced levels of interleukin-1- , interleukin-2, interleukin-6, interleukin-8 and tumor necrosis factor ~. Among these, interleukin-8, a potent neutrophil activating chemokine expressed by gastric epithelial cells, apparently has a central role. *H. pylori* strains carrying the *cag*-PAI induce a far stronger interleukin-8 response than *cag*-negative strains, and this response depends on activation of nuclear factor *k*B (NF-*k*B) and the early-response transcription factor activator protein 1.

H. pylori infection induces a vigorous systemic and mucosal humoral response. Yet, these antibodies cannot clear the infection but may contribute to tissue damage. Some *H. pylori* infected patients have an autoantibody response directed against the H+/K+–ATPase of gastric parietal cells that correlates with increased atrophy of the corpus. During specific immune responses, different subgroups of T cells emerge. These cells participate in mucosal protection and help distinguish pathogenic bacteria from commensals. Immature T helper (Th) 0 cells expressing CD4 can differentiate into two functional subtypes: Th1 cells, secreting interleukin-2 and interferon ¥ and Th2 cells, secreting interleukin-4, interleukin-5 and interleukin-10. Whereas Th2 cells stimulate B cells in response to extracellular pathogens, Th1 cells are induced mostly in response to intracellular pathogens. Because *H. pylori* is noninvasive and induces a strong humoral response, a Th2-cell response would be expected. Paradoxically, *H. pylori*-specific gastric mucosal T cells are of Th1 phenotype. Studies in gene-targeted mice have further showed that Th1 cytokines promote gastritis whereas Th2 cytokines are protective against gastric inflammation. This Th1 orientation may be due to increased antral production of interleukin-18 in response to *H. pylori* infection. This biased Th1 response, combined with Fas-mediated apoptosis of *H. pylori* specific T-cell clones, may favor the persistence of *H. pylori* (Suerbaum and Michetti, 2002).

Epithelial cell damage can also result from reactive oxygen or nitrogen species produced by activated neutrophils. Chronic inflammation also increases epithelial cell

turnover and apoptosis, which may be due to the combined effect of direct Fas-mediated contacts between epithelial and Th1 cells and interferon-¥. The expression levels of Fas, NF-*k*B, and mitogen associated protein kinases are, in turn, regulated by interleukin-1 (Suerbaum and Michetti, 2002).

3.5.3 Clinical outcomes of *H. pylori* infection

H. pylori is responsible for chronic gastritis and the majority of peptic ulcers. There is very strong evidence that *H. pylori* increases the risk of gastric cancer (Asaka *et al.*, 2001). *H. pylori* infection significantly increases the risk of gastric MALT lymphoma, and 72 to 98 percent of patients with gastric MALT lymphoma are infected with *H. pylori*. The role of *H. pylori* infection in non-ulcer dyspepsia remains controversial. An increased prevalence of *H. pylori* has been reported in this condition, but inconsistent long-term symptom relief has been observed with bacterial eradication in large, randomized trials (Suerbaum and Michetti, 2002). Concerning the relationship between *H. pylori* infection and gastro-oesophageal reflux disease (GORD), the debate is ongoing whether the infection confers protection, is harmful or whether both entities are independent. Epidemiological evidence is given for an increased prevalence of GORD and a decreased prevalence of *H. pylori* infection in the western world. The assumption derived from it is that *H. pylori* protects from GORD (Malfertheiner, 2000). *H. pylori* may be involved in the extra-gastrointestinal diseases. Though a lot of associations have been studied, at present it seems that *H. pylori* could play a causative role in a subset of patients with chronic idiopathic thrombocytopenic purpura and atherosclerosis (Nilsson *et al.*, 2005).

3.6 Laboratory diagnosis of *H. pylori* infection

Several diagnostic tests have been developed to assess the presence of *H. pylori* infection. They are usually classified as invasive and non-invasive tests.

3.6.1 Invasive or biopsy-based tests

These tests require gastric biopsies. These are still the gold standard for the diagnosis of *H. pylori* and are mainly recommended for patients with gastrointestinal symptoms and for clinical studies regarding symptomatic individuals. These tests include RUT, culture, histopathology, PCR and fluorescent in-situ hybridization (FISH).

i. Rapid Urease Test (RUT)

McNulty and Wise were the first researchers to use RUT in the diagnosis of *H. pylori* infection. These tests are simple, cheap and well-adapted for testing in the endoscopy suite. Though sensitivity of the test is affected by the number of bacteria in the sample, it is widely used and is highly specific when read at one hour. At 24 hours false positive result may be obtained due to other urease producing bacteria (Moayyedi *et al.*, 1998).

Principle

When a biopsy specimen containing *H. pylori* is introduced into a urea rich medium, the urease hydrolyses the urea into carbon dioxide and ammonia. The ammonium ion increases the pH and phenol red changes its color from straw yellow to dark pink.

Different types of urease media can be utilized like Christensen medium and urea-indole medium. Commercial kits are also available such as the CLO test (agar based tests) and PyloriTek (strip-based tests).

H. heilmannii, a bacterium present in the stomach is also urease positive but may not give a positive result, as the bacterial load is often limited (Megraud and Lehours, 2007).

Limitations of the urease test

A limit to the urease test is the bacterial load required to obtain a sufficient sensitivity. It is estimated that at least 10^5 bacteria are necessary for a valid result. This amount may not be present after the treatment failure which makes this test less advisable for post-eradication follow-up. Treatment with PPI may also jeopardize the result. By changing

the milieu where the bacteria are present, especially the antrum, PPI renders it inhospitable and the bacterial load decreases. In addition, PPI may also have anti-urease properties. The presence of intestinal metaplasia may also lead to false-negative result which also corresponds to an inhospitable environment for *H. pylori (*Megraud and Lehours, 2007).

ii. Culture

Specimens like gastric biopsy, gastric juice, blood, stool as well as liver biopsy have been used for culture. Among them, gastric biopsies are considered the best of specimens. It must be ensured that the patients did not receive antibiotics or antisecretory drugs, especially proton pump inhibitors (PPI). The influence of these drugs depends on the dose and length of treatment; a single dose is not as detrimental as a substantial acid suppression (Megraud and Lehours, 2007).

A single biopsy specimen taken from the antrum gives good sensitivity. As *H. pylori* may have a patchy distribution examination of multiple biopsy specimens increases the probability of detecting the organism. There are some rare cases where the infection lies only in the corpus, but usually, *H. pylori* is present in all sites (Megraud and Lehours, 2007).

Transport of biopsy specimens

H. pylori is a fragile microaerophilic organism which must be protected from desiccation, contact with oxygen and room temperature. Hence, these specimens must either be placed in a normal saline solution for short-term transport (4 hours maximum) or in a transport medium (e.g., Portagerm pylori) maintained at 4°C. If transport medium is used, culture can be delayed by 24 hours. The biopsy specimens can even be frozen at –70°C or in liquid nitrogen in a dry tube and transported to the laboratory (Megraud and Lehours, 2007).

Grinding of biopsy specimens

In most cases, the bacteria are not homogeneously distributed in the biopsy specimens. Grinding allows the uniform dispersion of the bacteria in the specimen. It can be performed in an electrical/mechanical grinder along with a small volume of broth (Megraud and Lehours, 2007).

Culture on solid media plates

As *H. pylori* is difficult to culture in broth, processed specimens are directly plated. The media components include an agar base, growth supplements and selective supplements. Most agar bases are satisfactory for growing *H. pylori*, e.g., brain heart agar and Columbia agar. The growth supplements that are generally used include blood or serum (5, 7, or 10%) that contains numerous nutrients (vitamins and oligoelements, etc.) to enhance *H. pylori* growth. To prevent the overgrowth of contaminating organisms like gram-positive cocci from oral cavity and enteric gram negative bacilli selective supplements are incorporated in the culture medium. Different selective supplements contain antimicrobial compounds like vancomycin or teicoplanin to inhibit gram-positive cocci; polymyxin, nalidixic acid, colistin, trimethoprim or cefsulodin to inhibit gram-negative rods; and nystatin or amphotericin B to inhibit fungi. Use of both a nonselective medium and a selective medium or even two different selective media helps to increase the sensitivity of the test. A critical point is to use fresh media (Megraud and Lehours, 2007).

Helicobacters are microaerophilic and capnophilic bacteria. Several systems can be used to achieve a microaerophilic atmosphere such as a microaerobic cabinet or an incubator with an adjustable gas level, to jars in which the adequate atmosphere is created with an automatic apparatus or with H_2-CO_2-generating packs. *H. pylori* growth is also possible in a candle jar but it takes longer time and results in small colonies. The optimum temperature for growth is 37°C. For primary culture, colonies may appear after 3 days and are at their optimum on day 4. However, the plates have to be incubated for 7- to 10-days to ensure the negative result. In contrast, subcultures only take 2 to 3 days (Megraud and Lehours, 2007).

Phenotypic identification

The growth of small, circular, smooth colonies observed after 3 to 4 days on the media plated with gastric biopsy specimens is an important criterion for *H. pylori* identification. The biochemical tests used to identify *H. pylori* are oxidase, catalase and urease tests (Old, 2006). Among tolerance tests utilized to differentiate *Campylobacter* species, *H. pylori* can grow with 2, 3, 5-triphenyltetrazolium chloride (0.4 and 1 mg/liter), sodium selenite (0.1%) and glycine (1%), but not with glucose (8%) and sodium chloride (3.5%). *H. pylori* is susceptible to cephalothin but resistant to nalidixic acid, with some exceptions (Megraud and Lehours, 2007).

Strain maintenance

Culture of *H. pylori* is difficult to maintain. Colonies can survive on plates for a week provided they are kept in a microaerophilic atmosphere at 4°C. Bacteria must be frozen at a low temperature (70°C freezer or liquid nitrogen) for long term storage. Different broth media have been used, always with a cryoprotective agent, such as glycerol, either in cryotubes or on beads. As the freezing-thawing process is always lethal for the bacteria, it is necessary to use bacteria in their exponential growth phase, as they are more likely to survive (Megraud and Lehours, 2007).

A positive culture is 100% specific. It allows antibiotic sensitivity testing and further characterization of *H. pylori* strains. Disadvantages of the method are the specific medium conditions for transportation as well as specific laboratory equipment. The sensitivity of the test may be affected by patchy distribution of *H. pylori* in the stomach (Glupczynski *et al.*, 1998b).

iii. Histopathological diagnosis

This diagnosis was one of the first to be applied to the detection of *H. pylori*. It was because of the histopathological observation of Warren and Marshall that *H. pylori* was looked for and finally cultured.

Biopsy specimens collected during endoscopy are immediately introduced into a fixative (10% formaldehyde). This fixative maintains the morphology of the bacteria. Several stains such as Giemsa, Warthin Starry stain, Genta can be used. Immunostaining with a specific *H. pylori* antibody can also be used. The presence of inflammation (lymphocytes) and especially inflammatory activity (polymorphs) is a clear hint for searching *Helicobacter* (Megraud and Lehours, 2007).

Histopathological detection can reach a sensitivity of 95% under optimal conditions, with the limits being the quality of the material and the pathologist's expertise. The specificity is also in the range of 95% (Megraud and Lehours, 2007).

iv. Fluorescent in-situ hybridisation

It is a new technique for the detection of *H. pylori*. It can detect not only *H. pylori* infection but also resistance to antibiotics and presence of *cagA*, avoiding both culture and PCR. Fluorescent-labeled oligonucleotides bind to common *H. pylori* antigen, macrolide resistance antigen or *cagA*. After hybridisation the bacteria can be observed by fluorescence microscopy. The accuracy of the method has been shown to be high (Russmann *et al.*, 2001).

v. Polymerase chain reaction

Gastric biopsies can also be investigated by PCR. In this method, *H. pylori* DNA is extracted from the study material. Then, the presence of specific genes is detected using gene-specific primers.

3.6.3 Non-invasive tests

Non-invasive tests are indirect tests that detect either antibodies to *H. pylori*, *H. pylori* antigens or *H. pylori* urease. Non-invasive tests are easy to perform and are not influenced by patchy distribution of strains, therefore these tests are used in epidemiological studies.

i. ^{13}C – urea breath test (UBT)

UBT detects active *H. pylori* infection and is based on the urease activity of *H. pylori*. A solution of labeled urea ingested by the patient is rapidly hydrolyzed by *H. pylori* urease in the stomach; the labeled CO_2 is absorbed by the blood and exhaled that is detected in the breath. If the patient is not infected, no labeled CO_2 is produced and most of the isotope is eliminated in urine without modification. Two isotopes are generally used to label urea viz., ^{13}C and ^{14}C (Megraud and Lehours, 2007). The advantage of the method is its ability to assess the eradication of the bacterium after treatment and the possibility of using it in epidemiological studies especially in children. The drawbacks of the method are the high cost and false-negative results in case of treatment with proton pump inhibitors.

ii. Serology

Anti *H. pylori* antibodies can be detected in serum and plasma of *H. pylori* infected patients. Enzyme-linked immunosorbent assays (ELISA) and immunoblot have been utilized for this purpose. The accuracy of serologic test depends upon the lab conditions and antigen used. The method must be standardized in every population to achieve good accuracy (Suerbaum and Michetti, 2002). However, it is unreliable in small children and can not assess therapeutic outcome, as antibodies to *H. pylori* persists several months after eradication of infection. But if analysed quantitatively could be used to assess the therapeutic outcome (Brown, 2000).

iii. Stool antigen assay

H. pylori antigens can be detected in stools of *H. pylori* infected patients using ELISA based on monoclonal and polyclonal antibodies. The main advantage of this method is that it is applicable in large epidemiological studies in children.

iv. Urine antibody assay

This method is simple and rapid and detects specific *H. pylori* IgG antibodies eliminated in urine. Both ELISA and immunoblotting have been used. Commercial tests have been

developed in Japan: a standard ELISA, Urinelisa and a rapid immunochromatographic test, Rapirun (Megraud and Lehours, 2007).

v. Salivary antibody assay

Detection of salivary *H. pylori* IgG antibodies is a simple diagnostic test. The best results are obtained when the serum titer is high as in duodenal ulcer patients or in children older than 5 years (Megraud and Lehours, 2007).

vi. Polymerase chain reaction

PCR can also be used to detect *H. pylori* DNA in saliva, urine, faeces and environmental samples.

3.7 Prevention and control of *H. pylori* infection

Since the exact sources, reservoirs and mode of transmission of *H. pylori* is still unknown, there are no current recommendations for control and prevention. Based on epidemiological studies indicating person-to-person transmission, it is likely that general improvements in hygiene standards such as hand washing and good household sanitation will decrease transmission.

H. pylori infection is curable with antimicrobial therapy. But the treatment has been found to be difficult because of its unique location in the gastric mucosa where the antibiotics are poorly secreted or rapidly inactivated by the acidity of the stomach. Although certain agents appear active against the organism *in vitro*, they have not been found effective *in vivo* (Megraud *et al*., 2001).

The two most widely used regimens are bismuth triple therapy (a combination of tetracycline, metronidazole and bismuth) and proton-pump inhibitor therapy (a proton-pump inhibitor plus clarithromycin and either amoxycillin or metronidazole). Recently, sequential treatment consisting of PPI plus amoxycillin followed by PPI plus

clarithromycin plus tinidazole has been shown to be better than the combination of a PPI plus amoxycillin and clarithromycin (Malfertheiner et al., 2006).

No vaccine is currently available to prevent or treat *H. pylori* infection. However, several candidate vaccines are beginning to show promise in animal models.

3.8 Emergence of antibiotic resistance in *H. pylori*

H. pylori is intrinsically resistant to glycopeptides, cefsulodin, polymyxins, nalidixic acid, trimethoprim, sulfonamides, nystatin, amphotericin B and cycloheximide. Wild-type strains are susceptible to β-lactams (except cefsulodin), fosfomycin, macrolides, aminoglycosides, tetracyclines, chloramphenicol, rifampins, fluoroquinolones, 5 nitroimidazoles, and nitrofurans.

H. pylori acquires resistance by mutation. The mechanism involves point mutations which are transmitted vertically while transformation may be possible if two strains are present simultaneously in the stomach (Megraud and Lehours, 2007).

Macrolides act by binding to ribosomes at the level of the peptidyl transferase loop of the 23S rRNA. *H. pylori* resistance is the consequence of point mutations at two nucleotide positions, 2142 (A2142G and A2142C) and 2143 (A2143G), which lead to a conformational change and a decrease in macrolide binding (Megraud and Lehours, 2007). The resistance pattern varies with geographical area. Clarithromycin resistance was reported in 12.9% of the isolates in the United States (Duck *et al.,* 2004), 44.7% of the isolates in India (Thyagarajan *et al.,* 2003) and 7.8% of the isolates in Hong Kong (Gu *et al.,* 2006). The essential risk factor for clarithromycin resistance has been found to be prior consumption of macrolides. Resistance rate is higher in children because these drugs are frequently prescribed for treatment of respiratory tract infections. The prevalence of secondary clarithromycin resistance is extremely high. A study carried out

in Germany reported secondary clarithromycin resistance in 66% of the cases (Heep *et al.*, 2000).

5-Nitroimidazoles have to be reduced inside the cell to be active. This activity requires nitroreductase enzyme which is coded by *rdxA* gene. Mutations in *rdxA* can render the protein ineffective (Hoffman *et al.*, 1996). Other proteins may also be involved in this reduction process, like the flavin oxidoreductase (*frxA*), while their role is more controversial (Marais *et al.*, 2003; Mendz and Megraud, 2002). In addition, a TolC efflux pump appears to play a role in resistance to this group of drugs (van Amsterdam *et al.*, 2005). Metronidazole resistance was reported in 25.1% of the isolates in the United States (Duck *et al.*, 2004), 77.9% of the isolates in India (Thyagarajan *et al.*, 2003) and 39.2% of the isolates in Hong Kong (Gu *et al.*, 2006). *H. pylori* resistance to metronidazole appears to be the consequence of the extensive use of 5-nitroimidazole for gynecological and dental infections in the developed countries while parasitic infections in the developing countries.

Amoxycillin hinders the peptidoglycan synthesis, especially by blocking transporters named penicillin binding proteins (PBP). The amoxycillin resistant *H. pylori* strains harbor mutations on the *pbp-1a* gene. The amino acid substitution Ser-414-Arg appears to be involved (Gerrits *et al.*, 2002), leading to a blockage of penicillin transport. Tolerance to amoxycillin has also been described, and the mechanism proposed was the lack of a fourth PBP, namely PBP-D (Dore *et al.*, 1999). Amoxycillin resistance was reported in 0.9% of the isolates in the United States (Duck *et al.*, 2004), 32.8% of the isolates in India (Thyagarajan *et al.*, 2003) while all the isolates were reported to be susceptible to amoxycillin in Hong Kong (Gu *et al.*, 2006).

Culture and susceptibility testing for *H. pylori* are not routinely performed which may partly be due to the difficulty of growing *H. pylori* or due to the unavailability of internationally agreed susceptibility testing methods for *H. pylori.* Also may be due to recent international consensus statements that refer management of *H. pylori* infection

in primary care, based on the use of non-invasive diagnostic tests (urea breath test, stool antigen test, or serology). The requirement for culture has been restricted to selected situations in which resistance is more likely to be encountered, e.g., for patients in whom previous therapies have failed (Megraud *et al*, 2001).

Phenotypic as well as genotypic methods have been used for determining the antibiotic resistance of *H. pylori*. Phenotypic methods involve agar dilution, broth dilution, breakpoint susceptibility testing, disk diffusion technique and E-test. Disk diffusion is the simplest, cheap and readily applicable method for antibiotic testing of *H. pylori* that can be used in routine practice.

CHAPTER – IV

4. MATERIALS AND METHODS

4.1 Materials

Materials required for this study are mentioned in Appendix-V

4.2 Methods

This study was a cross sectional one carried out at microbiology laboratory, Bir Hospital, Kathmandu. It was conducted from June to August, 2008.

Enrolled cases: 110 dyspeptic (OPD) patients attending gastroenterology department for upper gastro-duodenal endoscopy.

Excluded cases: Patients treated with Non-Steroidal Anti-Inflammatory Drugs, Bismuth compounds and antibiotics, with abnormal bleeding tests, allergic to anaesthetics, pregnant patients, abnormal liver function test and undergone gastro-duodenal surgery.

An informed consent was taken from each patient for biopsy sample.

A clinical profile of the patients including age, sex, upper gastrointestinal symptoms, underlying illness, history of alcohol consumption, smoking and medication was recorded before collecting the sample.

4.2.1 Endoscopic procedure

The patient was asked to lie on the left side with the chin tucked against the chest. The bite guard was placed securely between the teeth and the distal end of the endoscope was passed through the bite guard till it entered the stomach and then further inserted into the duodenum. The gastro-duodenal mucosa was examined for the presence of any lesion or ulcer. The endoscopic findings were noted (By the clinician).

4.2.2 Collection of sample

Biopsy forceps was inserted through the channel in the endoscope. Antral biopsy specimens were collected in case of gastritis cases. In case of ulcer, biopsy specimens

were collected from the adjacent mucosa (By the clinician).

4.2.3 Processing of the sample

One specimen was directly inoculated into the urease broth for RUT. The other specimen was placed into a sterile screw-capped container containing 0.2 ml of normal saline to maintain humidity. In the laboratory, specimens were ground with a glass homogenizer until the formation of a homogenate. Then it was cultured as well as gram-stained.

4.2.4. Rapid Urease Test

The urease broth with biopsy sample was then incubated at 37 °C. The result was recorded after 30mins, 1 hour, 2 hours and after 24 hours. The test was recorded as RUT positive when the colour of the broth changed to pink within 2 hours. If the test media turned pink after 24 hours, it was recorded as urease positive after 24 hours.

4.2.5 Culture

The homogenized specimen was inoculated into Skirrow's media and chocolate agar by spread plate technique. The agar plates were then incubated at 37 °C under microaerophilic condition using a Campy Gen pack (Oxoid, Hampshire, England). The plates were examined for the bacterial growth after 48 hrs, 4 days and 10 days. If no growth was observed the plates were discarded after 10 days.

Bacterial isolates were identified by standard microbiological techniques that included colony morphology, gram staining and biochemical tests (catalase, oxidase and urease tests).

The colonies of *H. pylori* were subcultured on 7% sheep blood agar plates until antibiotic sensitivity testing was performed.

4.2.6 Antibiotic Sensitivity Test

The bacterial isolates were tested against metronidazole, amoxycillin and clarithromycin by disk diffusion method.

Inoculum Preparation

A 3-day subculture of *H. pylori* was taken. Colonies were removed with a sterile cotton swab moistened in sterile nutrient broth and emulsified in a small volume of nutrient broth. The turbidity of this bacterial suspension was then matched with that of McFarland opacity standard of 4.

Inoculation and incubation

Thus standardized bacterial suspensions were inoculated onto the entire surface of the Mueller Hinton agar plates supplemented with 5% sheep blood with a swab and then it was allowed to dry. Antibiotic discs (metronidazole, amoxycillin and clarithromycin) were placed aseptically onto the surface. All the plates were then incubated at 37 °C under microaerophilic condition for 3 days.

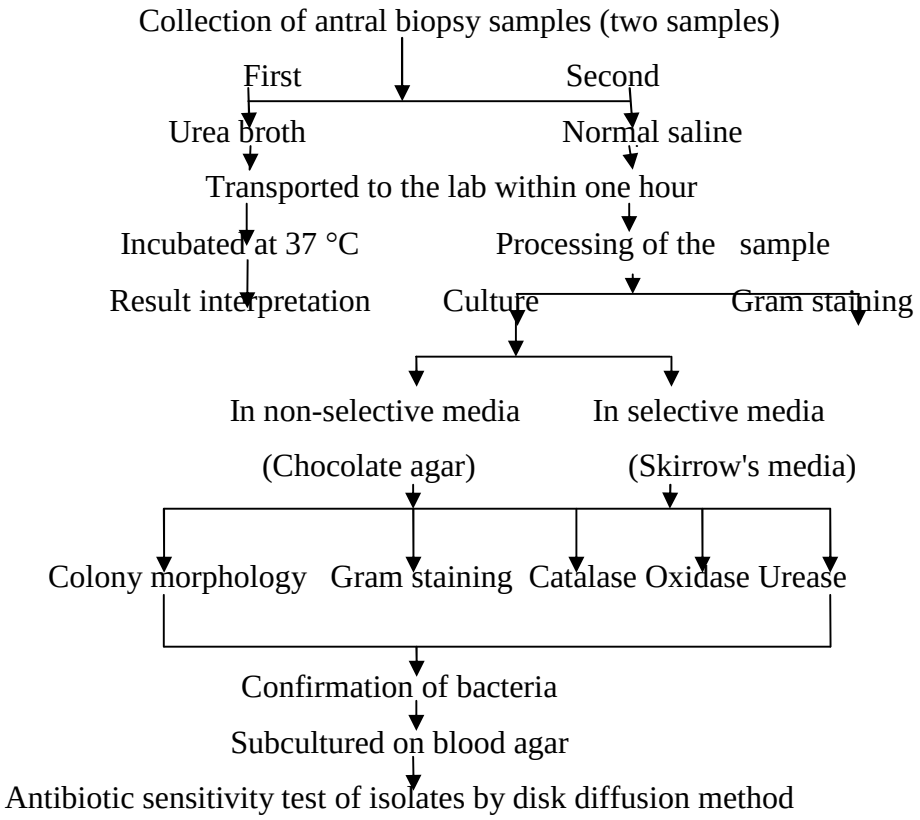
Reading and Interpretation of Results

The diameter of inhibition zone was noted and the results were interpreted as sensitive and resistant by comparing with the instructions provided by the manufacturer.

Table 2: Interpretation criteria for sensitive and resistant strains of *H. pylori*

Antibiotics (mcg)	Sensitive (S)	Resistant (R)
Metronidazole (5mcg)	>16mm	< 16mm
Amoxycillin (10mcg)	>16mm	< 16mm
Clarithromycin (15mcg)	>21mm	< 21mm

Flow chart of the procedure



CHAPTER - V

5. RESULTS

5.1 Positivity of biopsy samples for *H. pylori*

Among 110 patients, *H. pylori* positive results determined by culture and rapid urease test were 13.6% and 25.5% respectively.

Table 3: Distribution of *H. pylori* infection in dyspeptic patients

Total number of suspected cases	*H. pylori* positive cases	
	Culure	RUT
110	15 (13.6%)	28 (25.5%)

The final result was considered *H. pylori* positive if either the culture or the rapid urease test was positive. From the mentioned criteria, the overall prevalence of *H. pylori* infection among dyspeptic patients was 25.5% (28 of 110).

5.2 Gender-wise distribution of *H. pylori* infection

The male to female ratio for *H. pylori* infection was 1.03:1.

Table 4: Gender-wise distribution of *H. pylori* positive cases

Sex	Number of patients (n=110)	Detection of *H. pylori*	
		Culture (%)	RUT (%)
Male	58 (52.7%)	8 (13.8%)	15 (25.8%)
Female	52 (47.3%)	7 (13.5%)	13 (25%)

5.3 Age-wise distribution of *H. pylori* infection

The age of the patients ranged from 17 to 84 years. The mean age of the patients was 37.6±15. The prevalence of *H. pylori* infection was highest in the age group of 26-35 years.

Table 5: Age-wise distribution of *H. pylori* positive cases

Age group (In Years)	Number of patients	Prevalence of *H. pylori* infection *(%)* by culture	Prevalence of *H. pylori* infection *(%)* by RUT
17-26	26 (23.6%)	3 (11.5%)	4 (15.4%)
26-35	31 (28.2%)	6 (19.4%)	11 (35.5%)
35-44	24 (21.8%)	3 (12.5%)	6 (25%)
44-53	11 (10%)	1 (9.09%)	2 (18.2%)
53-62	9 (8.2%)	1 (11.11%)	3 (33.3%)
62-71	6 (5.5%)	1 (16.7%)	2 (33.3%)
71-80	1 (0.9%)	-	-
80-89	2 (1.8%)	-	-
Total	110	15 (13.6%)	28 (25.5%)

5.4 Association of *H. pylori* infection with endoscopic diagnosis

Table 6: Association of *H. pylori* infection with upper endoscopic findings

Endoscopic findings	Number of patients (%)	Prevalence of *H. pylori* (%) by culture	Prevalence of *H. pylori (%)* by RUT
Normal	11 (10%)	3 (27.3%)	5 (45.5%)
Gastritis	88 (58.2%)	7 (7.9%)	16 (18.2%)
Erosive Gastro-duodenitis	4 (3.6%)	1 (25%)	2 (50%)
Gastric Ulcer	3 (2.7%)	2 (66.7%)	2 (66.7%)
Duodenal Ulcer	4 (3.6%)	2 (50%)	3 (75%)
Total	110	15 (13.6%)	28 (25.5%)

The overall prevalence of *H. pylori* infection was higher in duodenal ulcer cases.

Apart from the 28 urease positive cases observed within 2 hours, additional 10 cases were positive after 24 hours but they were excluded while evaluating the prevalence as they were late urease positive in contrast to rapid activity of *H. pylori* urease.

5.5 Sensitivity, specificity, PVP and PVN of RUT

The sensitivity, specificity, PVP and PVN of RUT were 100%, 86%, 54 % and 100% respectively.

5.6 Antibiotic sensitivity test of *H. pylori*

Out of 15 culture positive cases, two of the isolates did not grow in subculture. Only 13 could be maintained in subculture. These 13 isolates were subjected to antibiotic sensitivity test.

Table 7: Results of antibiotic sensitivity test

S. No.	Metronidazole	Amoxycillin	Clarithromycin
H_6	R	S	S
H_{22}	R	S	S
H_{23}	S	S	S
H_{27}	S	S	S
H_{42}	S	S	S
H_{45}	S	R	S
H_{64}	R	S	S
H_{78}	R	S	S
H_{90}	S	R	S
H_{100}	R	S	S
H_{101}	R	S	S
H_{108}	S	S	S
H_{110}	R	S	S

Note: R- Resistant, S- Sensitive

Most of the isolates were resistant to metronidazole, 53.8% (7/13). Few were resistant to amoxycillin, 15.38% (2/13). All were sensitive to clarithromycin.

CHAPTER- VI

6. DISCUSSION AND CONCLUSION

6.1 Discussion

The present study was conducted with the objective to evaluate the prevalence of *H. pylori* infection in dyspeptic patients attending Gastroenterology Department, Bir Hospital. Most epidemiologic studies evaluating the prevalence of *H. pylori* infection are usually based on serological tests or UBT. However, biopsy-based methods are often used in hospital or clinical settings. In this study, the prevalence of *H. pylori* infection was determined using the biopsy-based methods, viz. RUT and culture.

Varieties of host factors and bacterial factors contribute to the pathogenesis of gastrointestinal diseases resulting from *H. pylori* infection. It is a major cause of morbidity in infected patients as it is associated with 80–100% of duodenal ulcers and 50-100% of gastric ulcers (Jyotheeswaran *et al.,* 1998). The disease has a low mortality. But it results in substantial human suffering and hence loss of manpower.

Among 110 dyspeptic cases, the overall prevalence of *H. pylori* was 25.5% (28/110) considering either RUT or culture positive result. Several previous studies have utilized either RUT alone (Yusuf *et al.,* 1999; Tumi *et al.,* 2007) or RUT and/or culture positive results to determine the overall prevalence of *H. pylori* infection (Nawapon *et al.,* 2005). The isolation of *H. pylori* from almost all positive RUT specimens (Jaup *et al.,* 2000) supports the utility of RUT in determining the overall prevalence. Additional 10 cases were urease positive after 24 hours but they were excluded as other contaminant intestinal bacteria can also produce urease after 24 hours. In various studies carried out in Nepal, the overall prevalence of *H. pylori* infection was 56.8% (Kawasaki *et al.,* 1998) using serology, 39.25% (Subedi, 2001) using histopathology and/or culture, 33.9% (Makaju *et al.,* 2005) using histopathology. In a study carried out in Pakistan, the overall prevalence was 45.66% (Zaman, 2006) using serology and [^{13}C] UBT. A study carried out in Sri Lanka reported the prevalence of 75.4% of *H. pylori* using PCR

(Fernando *et al.,* 2002*).* Similarly a study carried out in Bangladesh reported a seroprevalence of 92% (Ahmad *et al.,* 1997). The data aforesaid indicate lower prevalence of *H. pylori* infection in Nepal compared to other developing countries. It may be due to different social and cultural practices. The prevalence of *H. pylori* infection in symptomatic patients varies between regions of the same country or other parts of the world. Many variations, including studied populations, bacterial strains, geographic locations, the efficacy of diagnostic methods, environmental, and socio-economic factors may contribute which make it difficult to interpret existing data (Nawapon *et al.,* 2005).

The male to female ratio for the prevalence of *H. pylori* infection was 1.03:1. Several studies have reported similar rate of *H. pylori* infection in both genders (Kawasaki *et al.,* 1998; Subedi, 2001; Nawapon *et al.,* 2005). In some studies, the prevalence of *H. pylori* infection was higher in males (Makaju *et al.,* 2005; Chong *et al.,* 2008) while a study carried out in Pakistan reported higher prevalence in females (Zaman, 2006).

The prevalence of *H. pylori* infection was highest (35.5%) in the age group of 26-35 years. Subedi, 2001 reported higher prevalence in younger age group. Rai *et al.,* 2006 reported higher prevalence in 36-50 years. In developing countries, the prevalence of *H. pylori* infection is high in the 20-30 year age group and the prevalence of the infection varies between subpopulations within the same country, especially in relation to age (Malaty, 2007). In a study carried out in Brunei (a developing country), the overall prevalence of *H. pylori* infection was higher in the 30-39 year age group (Chong *et al.,* 2008).

H. pylori infection was higher in duodenal ulcer cases (75%). This finding shows similarity with the study of Subedi, 2001. A high association of *H. pylori* with peptic ulcer (duodenal ulcer, 75%; gastric ulcer, 56.4%) and gastritis (44.1%) was reported in Thailand by Nawapon *et al.,* 2005. Similarly, a study in Karachi, Pakistan reported that *H. pylori* was associated with 86% cases of chronic gastritis, 84.6% cases of duodenal

ulcers and 78.5% of gastric ulcers (Kazi *et al.,* 1990). In the present study, *H. pylori* was also detected in endoscopically normal stomach. It may be due to milder or an earlier infection (Hassan and Zaigham, 2007).

The present study revealed lower prevalence of *H. pylori* infection. It may be due to PPI treatment (collected data). With the increase in awareness about sanitation, hygiene and increased socio-economic status the prevalence rate may have decreased. However, further studies are required to relate these associations. Seroprevalence study reported higher prevalence of *H. pylori* infection in Nepal (Kawasaki *et al.,* 1998) as compared to biopsy-based studies (Subedi, 2001; Makaju *et al.,* 2005). This may be explained by the fact that serology not only reflects active infection but also past infection.

Culture positive cases were less compared to RUT positive cases. Although culture is highly specific and considered the gold standard technique its sensitivity is influenced by several factors, viz. sampling error due to patchy distribution of the organism in the gastric mucosa, contamination of the biopsy forceps, presence of oro-pharyngeal flora, transport condition, nature of media, atmospheric condition of the culture and also on the experience and preference of the lab.

Various selective as well as non-selective media are commercially available for the isolation of *H. pylori*. In this study, both non-selective (chocolate agar) and selective media (Skirrow's medium) were used. Selective medium contains antibiotics such as vancomycin, polymyxin B and trimethoprim which restrict the overgrowth of contaminating microorganisms. Non-selective medium at the same time allows the growth of some *H. pylori* strains that may be suppressed by the antibiotic supplements (Ansorg *et al.,* 1990; Hua *et al.,* 1998).

Since culture is considered the gold standard technique in the diagnosis of infectious disease sensitivity, specificity, PVP and PVN of RUT were calculated by comparing it with culture. The sensitivity, specificity, PVP and PVN of RUT were 100%, 86%, 54 %

and 100% respectively. The higher sensitivity and PVN may be due to the short time period utilized for interpreting the results of RUT.

McNulty *et al.*, 1989 reported the sensitivity and specificity of RUT to be 95% and 100% respectively. Goh *et al*., 1994 reported the sensitivity, specificity, PVP and PVN of RUT to be 96.6%, 99.2%, 99.3% and 96.2% respectively. Chomvarin *et al.*, 2005 reported the sensitivity, specificity, PVP and PVN of RUT to be 95.7%, 98.3%, 97.8% and 96.6% respectively.

H. pylori infection is a chronic infection and once acquired remains life long, unless treated with antibiotics. Humoral and tissue immune response of the host is unable to clear the infection. The treatment for *H. pylori* should be given only after the clinical diagnosis and proper indication for eradication. The National Institute of Health of USA recommends eradication of *H. pylori* in all patients with active peptic ulcer disease or a history of it and proved infection.

Antimicrobial treatment of *H. pylori* is difficult, as the bacteria are located below the mucus layer, adherent to gastric mucosa where the access of antimicrobial drugs is limited. Further acidic contents of the stomach may inactivate the drugs. Apart from these limitations, patient's non-compliance and emerging antibiotic resistance also contribute to treatment failure. Hence, pretreatment antibiotic susceptibility testing is necessary.

Antibiotics frequently prescribed for the treatment of *H. pylori* infection in Nepal are metronidazole, clarithromycin and amoxycillin. Resistance to these antibiotics widely varies between geographical regions and among subgroups within a population. Local prevalence of antimicrobial resistance determines the first line antibiotic to be included in a treatment regimen. In the context of Nepal, data regarding the antibiotic resistance must be collected from prior studies as culture and antibiotic sensitivity testing for *H.*

pylori are not routinely performed. This may be due to difficulty of culturing the bacteria, high cost, lack of validated techniques to be used in routine AST, etc.

In this study, antibiotic resistance among the *H. pylori* isolates was assessed by disk diffusion method as it is a simple, economical and readily applicable technique in routine practice. Three antibiotics, viz. metronidazole (5 mcg), amoxycillin (10mcg) and clarithromycin (15 mcg) were used. 53.8% of the isolates were metronidazole resistant, 15.38% were amoxycillin resistant while all were sensitive to clarithromycin. Further, none of the isolates were multi-drug resistant.

The previous study of Subedi, 2001 reported that 50% of the isolates were resistant to metronidazole, 6.6% were resistant to amoxycillin and 3.33% were resistant to clarithromycin. Comparing the result of this study with the prior work, it appears that metronidazole and amoxycillin resistance is increasing with all the isolates being sensitive to clarithromycin.

Studies in the United States reported the clarithromycin resistance among 10 to 15% and metronidazole resistance among 20 to 40% of *H. pylori* isolates (Duck *et al.,* 2004; Meyer *et al.,* 2000; Osato *et al.,* 2001). In Korea and Japan clarithromycin resistance was reported among 5.9% and 20% of the *H. pylori* isolates respectively (Kim *et al.,* 2001; Hiyama *et al.,* 2003). In developing countries, the prevalence of metronidazole resistance among *H. pylori* isolates was much higher, 50 to 80% (Nahar *et al.,* 2004, Torres *et al.,* 2001 and Wheeldon *et al.,* 2004) and was relatively rare in Japan, 9 to 12% (Perez *et al.,* 2002). The prevalence of *H. pylori* resistance to amoxycillin was very low (1%), except in a few countries like South Korea (Kim *et al.,* 2001).

Metronidazole resistance seems to be more prevalent in our country. The possible reason may be due to previous consumption of this antibiotic in amoebiasis, giardiasis, other protozoan infections and gynecological problems. Different mechanisms of metronidazole resistance in *H. pylori* have been put forward. Mutations in *rdx A* gene

and *frx A* gene as well as involvement of Tol C efflux pump appeared to play a role in resistance to this antibiotic.

Resistance to amoxycillin may be due to common consumption of this antibiotic for other reasons (respiratory tract infections, urinary tract infections etc.). Mutation in *pbp 1a* gene that codes for PBP has been implicated in amoxycillin resistant *H. pylori* strains.

This *in vitro* study showed that clarithromycin is the most sensitive drug in the treatment of *H. pylori* infection. However, it requires more investigation not only on antibiotic sensitivity testing but also on clinical outcome (*in vivo*) evaluation of the patient after treatment.

As most of the isolates were resistant to at least one of the frequently prescribed antibiotics, pretreatment culture and antibiotic susceptibility testing of *H. pylori* helps the clinicians to choose appropriate therapy. Further, more effective regimens are urgently needed as this organism predisposes to various serious conditions like peptic ulcer and gastric cancer.

6.2 Conclusion

The overall prevalence of *H. pylori* infection was 25.5% among dyspeptic patients referred for endoscopy in Bir hospital. RUT facilitated the detection of higher percentage of *H. pylori* infection than culture. The male to female ratio for *H. pylori* infection was 1.03:1. *H. pylori* infection rate varied with age. Further, *H. pylori* infection rate was higher in the duodenal ulcer cases. The sensitivity, specificity, PVP and PVN of RUT were high. The majority of the isolates were resistant to metronidazole, few to amoxycillin while all were sensitive to clarithromycin.

CHAPTER - VII

7. SUMMARY AND RECOMMENDATIONS

7.1 Summary

1. This study was conducted in microbiology laboratory, Bir Hospital, Kathmandu from June to August, 2008 with the objective to determine the prevalence of *H. pylori* infection. Antral biopsies were collected from 110 dyspeptic patients visiting Gastroenterology Department, Bir Hospital.

2. Two methods were used to assess the prevalence, viz. RUT and culture. 15 (13.6%) cases were positive by culture while 28 (25.5%) cases were positive by RUT. The overall prevalence was 28 (25.5%) by considering either RUT or culture positive cases.

3. Out of 110 patients, 58 (52.7%) were males and 52 (47.3%) were females. The male to female ratio for *H. pylori* infection was 1.03:1.

4. The prevalence of *H. pylori* infection was higher (35.5%) in the age group of 26-35 years.

5. *H. pylori* infection rate was higher in duodenal ulcer cases, i.e. 75%.

6. Among 15 culture positive cases, only 13 of the bacterial isolates could be maintained in subculture. These 13 isolates were subjected to antibiotic sensitivity test. Among them 7 (53.8%) were resistant to metronidazole, 2 (15.38%) were resistant to amoxycillin while all the isolates were susceptible to clarithromycin. None of the isolates were MDR strains.

7. The sensitivity, specificity, PVP and PVN of RUT were 100%, 86%, 54 % and 100% respectively.

7.2 Recommendations

Following recommendations can be put forward on the basis of this research:

1. As *H. pylori* infection rate was found to be higher in the age group of 26-35 years, studies targeted at this age group are needed that can provide more information about sources, risk factors and transmission of *H. pylori*.

2. Studies determining Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of metronidazole against *H. pylori* are needed that will guide the clinicians in therapy.

3. In a developing country like Nepal where the bacterial culture facility is extremely limited due to high cost as well as special transportation and laboratory conditions needed for the isolation of *H. pylori*, rapid urease test should be preferred for the diagnosis of clinically suspected patients.

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